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MEMOIRS OF THE DEPARTMENT OF AGRICULTURE IN INDIA

AZOTOBACTER AND NITROGEN FIXATION IN INDIAN SOILS

BY

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AGRICULTURAL RESEARCH INSTITUTE, PUSA

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PREFACE.

This memoir is an account of some preliminary observations on the problem of nitrogen fixation in Indian soils.

More detailed investigations are now being carried out, with special reference to the possibility of increasing the amount of nitrogen fixation in the soil by methods applicable in agricultural practice.

PUSA,

J. H. WALTON.

February 10th, 1915.

INTRODUCTION.

THE enormous area of cultivated soil in India which is never likely to receive any addition of nitrogenous manures but is yet the only source of nourishment and livelihood of millions of cultivators, makes the nitrogen problem in this country of paramount importance from the point of view of the transference of this element from the air to the soil by various natural agencies. The importance of leguminous crops in this respect cannot be overestimated, but in actual practice the fact remains that enormous quantities of nitrogen are annually going out of the country in the form of agricultural exports, whereas the amount actually added to the soil by the cultivation of *Leguminosæ* cannot be considered sufficient to replace this loss, nor is there any importation of artificial nitrogenous manures in quantities worthy of consideration. Another source of soil nitrogen remains, namely, the non-symbiotic organisms such as *Clostridium* and *Azotobacter* which, so far as our knowledge at present carries us, are largely responsible for the present state of fertility of soils all over the world.

Clostridium works under semi-anaerobic conditions and it is probable that the nitrogen obtained from the air by this organism does not add any considerable quantity to the soil supply.

Azotobacter, however, has been shown to exist and perform its valuable work in cultivated soils all over the world, and although we have at present insufficient data upon which to base any accurate conclusions as to the actual quantities of nitrogen obtained from the air and added to the soil supply by this organism, there is good reason for supposing that it is sufficiently large to support a considerable growth of natural vegetation continuously. This result is no doubt due to the continuous nature of the action of this organism which, as the author of the following paper shows, goes on in India at all times of the year, so that although the actual rate of fixation is small the annual increment may be considerable.

The optimum conditions for growth and nitrogen fixation by *Azotobacter* are obviously of importance to the agriculturist, and the object of the work, the preliminary stages of which are described in this paper,

was to ascertain whether *Azotobacter* is of universal occurrence in Indian soils, to determine the optimum conditions for the exercise of its specific function, and how far agricultural practice might be modified so as to take advantage of its nitrogen-fixing power.

C. M. HUTCHINSON,
Imperial Agricultural Bacteriologist.

AZOTOBACTER AND NITROGEN FIXATION IN INDIAN SOILS.

BY

J. H. WALTON, B.A., B.Sc.,

Supernumerary Agricultural Bacteriologist, Pusa.

AZOTOBACTER IN INDIAN SOILS.

The results of many investigations carried out in Europe and America show that the micro-organism 'Azotobacter' is able to assimilate the free nitrogen of the air, and thus indirectly make it available for the higher plants.

In order to determine whether this organism is present and similarly active in the soils of India, investigations were started at Pusa in 1913.

The method adopted was that used by Ashby (*Journal of Agricultural Science*, Vol. II, p. 38).

A nutrient solution was made of the composition :—

Mannite	12 or 20 gms.
Monopotassium phosphate	0·2 gm.
Magnesium sulphate crystal	0·2 ,,
Sodium chloride	0·2 ,,
Calcium sulphate	0·1 ,,
Distilled water	1000 c.c.

The phosphate was dissolved separately, and made just alkaline to phenol phthalein by the addition of decinormal sodium hydrate. 100 c.c. of the solution with half a gram of calcium carbonate was put into each of ten Erlenmeyer flasks of 250 c.c. capacity and sterilized at 130°C. for fifteen minutes.

One gram of Pusa field soil was added to a flask of the medium, which was then placed in the incubator at 30°C.

At the end of two days numerous gas bubbles were visible in the medium, and at the end of three or four days the surface of the medium was covered with a thin greyish wrinkled film. The film gradually thickened and turned

brownish. In some cases black spots appeared on the brown film, which itself occasionally turned quite black. Usually the film turned a deep brown colour, and was then easily detached from the wall of the flask and broken up by slight agitation.

Duplicate flasks, one exposed to light, the other kept in the dark, showed that the pigment developed more rapidly in the light. On continued exposure to light, a green algal growth gradually covered the broken masses of the film and the wall of the flask below the level of the solution.

An examination of the young film showed it to consist of oval organisms, and cocci, single or united in pairs, mixed with various rod-shaped organisms, hyphae of fungi, etc. (Pl. I, fig. 1.).

The oval organisms and cocci were 1·5μ—2μ in diameter, and were actively motile, they stained golden yellow to golden brown with iodine, and generally resembled *Azotobacter*.

A set of three flasks of the medium was taken, 1 gm. soil as inoculum added to each, one flask sterilized and the other two incubated at 30°C for twenty days, at the end of which the nitrogen in the three flasks was estimated by the Kjeldahl-Gunning method. It was found that the duplicate flasks contained 13·16 and 12·46 mgms., or a mean of 12·8 mgms. of nitrogen more than the control. As the flasks contained 2 gms. mannite each, this gives a fixation of nitrogen of 6·4 mgms. per gram of mannite. The ability to fix nitrogen being shown, the medium was varied in order to find, if possible, one more favourable for nitrogen fixation. The length of the incubation period was also varied, to find the best period.

To find the best length of time for incubation, three sets of duplicate flasks were inoculated and incubated for 7, 10 and 14 days respectively.

Length of incubation period.	Amount of Nitrogen fixed. Mgms.
7 days	6·72
10 „	14·90
14 „	14·60

No mannite was found in the culture medium after fourteen days' incubation.

Ten to fourteen days therefore appears to be the best length of incubation period. The period adopted for all subsequent estimations of nitrogen fixation in impure cultures was fourteen days.

A comparison was made at the same time between the nutrient solution of Ashby and those recommended by Bottomley (*Ann. Bot.*, XXVI, p. 875).

Compared with the amount of 14·6 mgms. obtained after fourteen days with Ashby's solution, these gave only 7·84 mgms. and 10·5 mgms. respectively.

Effect of varying the amount of mannite in the solution.

Three lots of nutrient solution were made up, containing 10, 12 and 20 grams mannite per litre respectively. Duplicate flasks from all three were inoculated with soil, incubated for 14 days, and the amount of nitrogen estimated.

Medium	Mgms. Nitrogen fixed per gm. mannite in solution
(*i*) With 10 gms. mannite per litre	8·4
(*ii*) 12	7·2
(*iii*) 20	7·0

The solution containing 10 gms. mannite per litre therefore is most economical under the conditions of the experiment, as a higher fixation of nitrogen per gm. mannite is obtained with it than with the other two solutions.

For later work, ten grams of mannite per litre was always taken in making up the nutrient medium.

Variations in the inorganic constituents of the medium.

(*a*) Omission of NaCl and (*b*) substitution of dipotassium phosphate for monopotassium phosphate.

	Mgms. Nitrogen fixed	
	1st set	2nd set
Ordinary medium ..	9·73	9·24
Medium (*a*) ..	8·96	9·03
Medium (*b*) ..	9·17	8·89

Hence these two variations interfere slightly with the efficiency of the medium.

(*c*) Addition of basic slag.

½ gm. basic slag was added, in addition to the other constituents.

In medium without slag 9·59 mgms. nitrogen were fixed.

„ „ with „ 9·52 „ „ „ „

Basic slag is therefore without influence on the fixation of nitrogen in cultures inoculated with Pusa soil.

(*d*) Addition of ferric chloride.

The addition of a drop of ferric chloride solution, sufficient to give a faint yellow tinge to the medium, depressed the fixation of nitrogen.

	Mgms. Nitrogen fixed
Without ferric chloride	8·40
With „ „	6·16

(*e*) Magnesium carbonate.

Substitution of 0·25 gm. $MgCO_3$ for the 0·5 gm. $CaCO_3$ raised the fixation of nitrogen from 9 mgms. to 9·4 mgms. The addition of 0·25 gm. or 0·5 gm. $MgCO_3$ as well as the 0·5 gm. $CaCO_3$ was without influence.

Pusa soil itself contains over 30% of calcium carbonate, so the slight increase of nitrogen fixed in the first case may be due to the joint influence of the calcium carbonate in the soil and the magnesium carbonate added, but the effect is in any case very small.

(*f*) Addition of ammonium sulphate.

Medium with the addition of ammonium sulphate sufficient to add 4 mgms. of combined nitrogen per 100 c.c. medium was compared with the original medium.

	Mgms. Nitrogen fixed
Medium without $(NH_4)_2SO_4$ ..	9·59
„ with $(NH_4)_2SO_4$ ..	6·44

he addition of ammonium sulphate therefore decreases the amount of nitrogen fixed.

Seasonal Variation in Nitrogen Fixation.

In November 1913 duplicate flasks with a control were inoculated with 1 gm. Pusa soil from plots which were kept free from weeds, the control sterilized, and the flasks incubated at 30°C. for 14 days, at the end of which the nitrogen was estimated.

This was done as far as possible every week for a year. The results, the mean values from the duplicate flasks, are given in Table I, the mean fixation in the month in Table II, and the figures in Table II shown graphically in Fig. 1.

Table I.

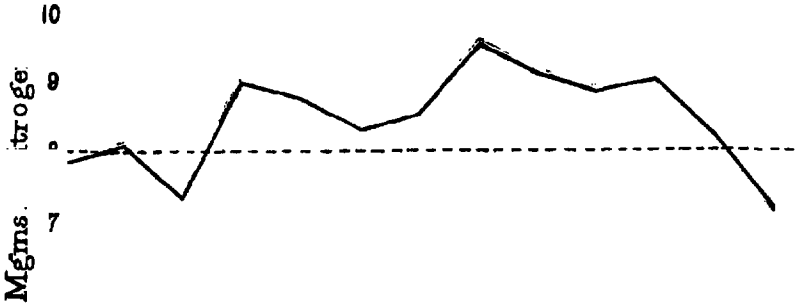
Date of inoculation	Mgms. N. fixed	Date of inoculation	Mgms. N. fixed
November 7	7·53	May 1	7·84
„ 14	7·11	„ 8	8·89
„ 21	8·40	„ 15	8·19*
„ 28	8·26	„ 22	8·47
December 5	7·84	„ 29	9·03
„ 12	8·28	June 5	9·66
„ 19	8·16	„ 12	9·24
„ 26	7·91	„ 19	....
January 2	7·28	„ 26	9·29
„ 9	8·40	July 3	8·68
„ 16	6·93	„ 10	10·01
„ 23	7·07	„ 17	9·52
„ 30	7·14	„ 24	8·12
February 6	7·49	„ 31	9·03
„ 13	9·59	August 7	8·75
„ 20	9·03	„ 14	8·40
„ 27	9·73	„ 21	8·72
March 6	....	„ 31	9·38
„ 13	8·19	September .. 11	8·30
„ 20	9·24	„ .. 18	9·74*
„ 27	....	„ .. 25	8·77
April 3	8·12	October .. 2	11·19
„ 10	8·47	„ .. 9	8·40*
„ 17	8·82	„ .. 16	7·17
„ 24	7·81	„ .. 23	6·93
		„ .. 30	7·42
		November .. 7	7·21

* Only one flask.

Table II.

Month	Mean fixation	Month	Mean fixation
November .. 1913	7·82	May 1914	8·48
December .. „	8·05	June .. .. „	9·40
January .. 1914	7·36	July .. .. „	9·07
February .. „	8·96	August .. „	8·81
March .. .. „	8·72	September .. „	8·94
April .. .. „	8·30	October .. „	8·22

It may be seen from Fig. 1, that nitrogen fixation is at its lowest in the months October to January, and at its maximum in June to September.



Nov. Dec. Jan. Feb. Mar. Apl. May. June. Jly. Aug. Sep. Oct. Nov.

FIG. 1.

Low fixation coincides with the drying of the soil and lowering of the temperature on the arrival of the cold weather. The abundant, but not excessive, moisture and the fairly high temperature of the rainy season at Pusa are accompanied with high fixation of nitrogen.

Influence of temperature of incubation on nitrogen fixation in the liquid medium.

In December 1913 three sets of duplicate flasks were incubated for 14 days at 20°C., 30°C. and 37°C. respectively.

At 37°C. the film developed very badly, and was very thin; at 20°C. the film developed rather more slowly than at 30°C.

Temperature	Mgms. Nitrogen fixed
20°	8·96
30°	7·84
37°	6·02

Further experiments were carried out throughout the year, comparing the nitrogen fixation at 20°C. and 30°C.

The results are given in Table III.

TABLE III.

Date of inoculation	Temperature	Mgms. N. fixed	Date of inoculation	Temperature	Mgms. N fixed
December .. 19	20°	3·78	June .. 26	20°	8·68
	30°	8·16		30°	9·20
January .. 2	20°	6·86	July .. 10	20°	9·10
	30°	7·28		30°	10·01
April 3	20°	8·47	„ .. 17	20°	8·26
	30°	8·13		30°	9·52
„ 17	20°	6·58	„ .. 24	20°	6·86
	30°	8·82		30°	8·12
May .. 1	20°	8·26	August .. 7	20°	7·77
	30°	7·84		30°	8·40
„ 15	20°	7·84	October .. 16	20°	6·86
	30°	7·49		30°	7·17
„ 29	20°	8·54	November .. 7	20°	7·40
	30°	9·03		30°	7·21
June 12	20°	9·17			
	30°	9·24			

Thus in eleven cases nitrogen fixation is greater at 30°C. than at 20°C., and in the remaining six cases it is less at 30°C. than at 20°C. In general, therefore, it may be taken that 30°C. is a more favourable temperature of incubation than 20°C.

NITROGEN FIXATION IN SOIL.

Lots of 100 gms. Pusa soil with different amounts of carbohydrate added, and with the moisture content made up to 16%, were incubated at 30°C. for three weeks, and at the end of that time the nitrogen content was estimated.

	Mgms. N. per 100 gms. soil	Gain of nitrogen in mgms.
Soil original	44·1	—
„ after incubation	46·6	2·5
„ + 1 gm. dextrose, after incubation.	49·7	5·6
„ + 2 gms. „ „ „ ..	50·8	6·7
„ + 1 gm. mannite „ „ ..	49·0	4·9
„ + 2 gms. „ „ „ ..	48·3	4·2

In November 1913, three plots were taken, and the nitrogen content of the soil in each to a depth of 6 inches was estimated.—

Plot I. 6′ × 6′ was left untouched.

II. 6′ × 6′ was cultivated weekly to a depth of six inches.

III. 6′ × 3′ had 2 lbs. cane sugar mixed with the surface soil, and was cultivated in the same manner as plot II.

After ten weeks, the nitrogen was again estimated.—

	Mgms. of Nitrogen per 100 gms. of soil		
	At the beginning	At the end	Gain or loss (+ or −)
Plot I	65·2	64·4	− 0·8
II	63·8	66·5	+ 2·7
III	71·4	81·9	+10·5

These experiments show that suitable carbohydrate food and thorough cultivation assist the soil bacteria in accumulating nitrogen.

Distribution of *Azotobacter*.

Soil samples from many localities were examined for the presence of *Azotobacter*, which was found in those from Pusa, Cawnpore, Darjeeling, Sind, Bangalore, Bowringpet, Jalarpet, Ellore, Naupada, Tuni and Walajah Road.

At Pusa on two occasions soil samples were taken to a depth of three feet. In the one case *Azotobacter* was found at all depths to three feet, in the other it was found at two feet but not at three feet below the surface.

Pure Culture.

To obtain a pure culture of *Azotobacter* from an impure liquid culture, a portion of the film and liquid is removed by a platinum loop, diluted in 10 c.c. sterile water, and a loopful of the suspension plated in mannite agar. The mannite agar contains the same ingredients as the liquid medium, with the addition of 20 gms. agar per litre.

The plate is incubated at 30°C. In two or three days glistening, milky colonies of *Azotobacter* may be picked out. A slant is made from one of those which on microscopic examination appears to be pure ; a good growth is usually obtained in 24 hours. Further plates are made from the slant and a pure culture may be obtained.

After having some experience, once plating from the first slant generally is sufficient to obtain a pure culture. It was found that a pure culture was most readily obtained when a loopful was taken from the impure culture

immediately before the appearance of the surface film. Microscopic examination of a stained preparation of the impure culture, before the appearance of the film, is of assistance in judging the best time to plate. When the film was more than seven days old, considerable difficulty was often experienced in obtaining a pure culture. Inoculating a fresh flask with the soil and using this new culture was found to save time and trouble.

Nitrogen Fixation in Pure Culture.

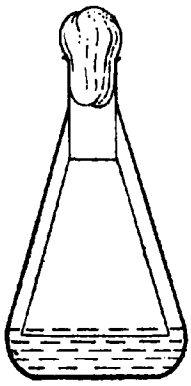


Fig. 2.

Duplicate flasks of mannite solution were inoculated with a pure culture of *Azotobacter* from Pusa soil and incubated for 23 days.

Another pair had a strip of filter paper cut into the shape shown in Fig. 2, and weighing 0·7 gm. suspended in each flask and dipping into the liquid.[1] These flasks and filter papers were sterilized, inoculated, and incubated for 23 days.

	Nitrogen fixed
Without filter paper	1·89 mgms.
With „ „	6·44 „

The beneficial effect of the filter paper was very strongly marked. In the flasks without the filter paper little growth was observed, while in the flasks with filter paper abundant growth occurred on the filter paper just above the level of the liquid and in the liquid itself flocculent masses floated.

Effect of addition of humus.

Humus was extracted from Sepinjuri Bheel soil with dilute ammonia, precipitated with dilute acid, washed with water, and allowed to dry.

	Nitrogen fixed in 21 days
Solution with filter paper	5·6 mgms.
„ „ „ „ + 2 gm. humus from Bheel soil	7·85 „
Increase due to addition of humus	2·25 „

Hence the addition of such humus is favourable to nitrogen fixation.

Substitution of $MgCO_3$ for $CaCO_3$.

	Nitrogen fixed in 21 days
Mannite solution + filter paper + $CaCO_3$	.. 6·72
+ 0·2 gm. $MgCO_3$	5·74

[1] Söhngen, *Cent. für. Bakt.* Abt. II, Vol. XXXVIII, p. 630. Söhngen however used 10 gms. filter paper.

Calcium carbonate is therefore superior to magnesium carbonate for promoting nitrogen fixation in pure culture.

It was noted that the brown pigment developed much earlier in the cultures containing $MgCO_3$ than in those with $CaCO_3$.

Addition of soil.

Two gms. of soil were added to some of the flasks of medium and the whole sterilized. The flasks were inoculated with *Azotobacter* and incubated for 22 days.

	Nitrogen fixed
Solution with filter paper	6·51 mgms.
,, ,, ,, ,, + 2 gm. soil ..	5·88 ,,
,, ,, 2 gm. soil	6·30 ,,

The addition of soil was equivalent to the addition of the filter paper. The smaller amount of nitrogen fixed when both the filter paper and the soil were added is rather noteworthy, for the cultures remained pure.

Addition of nitrogenous compounds.

Amounts of various nitrogen compounds containing 4 mgms. of combined nitrogen were added to duplicate flasks of the medium containing filter paper; these were sterilized and inoculated with *Azotobacter*, and incubated for 21 days.

	Nitrogen fixed
Mannite solution + filter paper	5·60 mgms.
,, ,, + ,, ,, + urea ..	4·97 ,,
,, ,, + ,, ,, + ammn. sulphate	4·97 ,,
,, ,, + ,, ,, + peptone ..	5·18 ,,
,, ,, + ,, ,, + acetamide ..	7·07 ,,
,, ,, + ,, ,, + asparagin ..	6·89 ,,
,, ,, + ,, ,, + casein ..	6·02 ,,

The experiment was repeated with acetamide and asparagin, but the considerable increase in N. fixation obtained in the first experiment did not occur in the second.

	Nitrogen fixed
Mannite solution + filter paper	5·95 mgms.
,, ,, + ,, ,, + acetamide ..	6·16 ,,
,, ,, + ,, ,, + asparagin ..	5·88 ,,

The addition of these nitrogenous substances has therefore but a small influence on the fixation of nitrogen by *Azotobacter*.

Influence of basic slag.

The effect of the addition of 0·2 gm. basic slag to the medium, with and without filter paper, was examined.

	Mgms. N. fixed in 25 days
Mannite solution + filter paper	6·16
„ „ + „ „ + 0·2 gm. basic slag	7·14
„ „ + 0·2 gm. basic slag.. ..	8·71

Basic slag therefore was decidedly beneficial.

A fresh culture of *Azotobacter* was isolated from Pusa garden soil and the basic slag was found in one case to have no influence on nitrogen fixation by this strain of *Azotobacter*, which fixed nitrogen freely without the aid of filter paper.

	Mgms. N. fixed in 3 weeks
Mannite solution only	6·86
„ „ + 0·5 gm. basic slag ..	6·86

This was repeated, using 0·2 and 0·5 gm. basic slag.

	Mgms. N. fixed in 3 weeks	
Mannite solution only ..	..	7·07
+ 0·2 gm. basic slag	.. { 5·32 / 4·62 }	4·97
+ 0·5 „ „ „	.. { 4·34 / 3·08 }	3·71

In this case the basic slag had an adverse influence on the nitrogen fixation.

Later, media with and without basic slag were inoculated with *Azotobacter*, from Sind soil, and incubated for three weeks, when the nitrogen content was determined.

	Nitrogen fixed
Medium without basic slag ..	6·12 mgms.
„ with 0·2 gm. basic slag ..	8·05 „

This shows that basic slag has a beneficial effect on nitrogen fixation by the Sind *Azotobacter*.

NITROGEN FIXATION BY *AZOTOBACTER* SPECIES FROM DIFFERENT LOCALITIES.

Duplicate flasks containing the medium were inoculated with strains of *Azotobacter* isolated from soils from different places. The amount

of nitrogen fixed after three weeks' incubation is given in the table below :—

Locality	Mgms. N. fixed	
Pusa ..	7·56 7·28	7·42
Cawnpore	6·72 6·44	6·56
Bangalore	3·50 3·50	3·5
Darjeeling	4·34 2·94	3·64
Nagpur	6·04 ——	6·04
Sind ..	6·16 6·16	6·16

As the culture of *Azotobacter* from Bangalore and Darjeeling fixed rather small amounts of nitrogen, a further series of flasks was inoculated three weeks after the first experiment, and further flasks were inoculated with the Nagpur variety two months after the first inoculation. The amount of nitrogen fixed is given below :—

Darjeeling	2·10 ——	2·10
Bangalore	2·80 2·38	2·59
Nagpur	5·04 4·40	4·72

These figures are smaller than the previous ones, showing that by repeated culture on agar slants these three varieties lose in nitrogen-fixing power. The Pusa variety maintains its nitrogen-fixing power without any diminution.

Morphology, Young Cultures, 24 hours old.

Azotobacter, Pusa.

Rods $1{\cdot}5\mu \times 2{\cdot}5\mu$ to 4μ. The longer rods occasionally are constricted, an indication of the beginning of division. Also cocci 1μ to $2\ \mu$ diameter, commonest $1{\cdot}5\mu$ to 2μ. Actively motile. (Pl. I, fig. 2.)

Azotobacter, Cawnpore.

Very like the Pusa variety. Rods $1{\cdot}5\mu \times 2\mu$. Cocci mostly $1{\cdot}5\mu$ but varying from $1{\cdot}25\mu$ to 2μ. Actively motile. (Pl. I, fig. 9.)

Azotobacter, Darjeeling.

Mostly cocci 2μ—3μ diameter. Also stout rods, 2μ × 2·5μ—3μ, and cocci less than 2μ in diameter. Motility. In the hanging drop a few individuals and diplococci may be seen moving sluggishly, and occasionally one may move vigorously. (Pl. II, fig. 1.)

Azotobacter, Bangalore.

The commonest forms are cocci 2μ in diameter, and diplococci.

Motility. In the hanging drop, a few individuals may be seen moving sluggishly. (Pl. II, fig. 3.)

Azotobacter, Nagpur.

Chiefly long rods 1·5μ × 3·75μ—5μ, which divide into oval individuals 1·5μ × 2μ—3μ. Sluggishly motile. (Pl. II, fig. 5.)

Azotobacter, Sind.

Oval rods 1·5μ × 2μ—2·5μ, also rods 1·5μ × 3μ—4μ, often with a constriction, the beginning of division. Actively motile. (Pl. II, fig. 8.)

In old cultures, all varieties take the coccus form. The size of individuals in the same culture varies considerably, as illustrated in Pl. I, fig. 4.

GROWTH IN VARIOUS MEDIA.

1. *Mannite Agar.*—Growth on mannite agar is rapid for all the varieties.

Pusa.—Growth grey and milky. The edges are watery. With age, it becomes more opaque and slightly brownish. On mannite agar plates the colonies are milky, translucent, with a margin watery or pale milky. (Pl. III, fig. 1.)

Cawnpore.—Very similar to Pusa variety, except that the margin is not watery, but milky, slightly less dense than the middle. Plate colonies, similar to Pusa except for margin. (Plate III, figs. 3 and 4.)

Bangalore.—Abundant watery growth. Plate colonies large, watery, 1—1·5 cm. diameter. (Pl. III, fig. 2.)

Darjeeling.—Thick creamy white growth. Plate colonies have nucleus, more opaque than margin. (Pl. IV, figs. 1 and 2.)

Sind.—Growth transparent milky, later becoming translucent with centre and margin. Even milky growth on plate. (Pl. IV, fig. 3.)

Nagpur.—Creamy growth, colonies on plate large and thick, very quickly acquire the radial and concentric structure. (Pl. IV, fig. 4.)

2. *Ordinary Agar.*—Pusa, Cawnpore and Bangalore.—Growth very thin and tough.

Darjeeling and Nagpur.—Growth opaque, thick, drawing out into threads. Better growth in Darjeeling than Nagpur.

Sind.—Thin, translucent, tough, spreading more than any other variety, except Darjeeling.

3. *Bouillon.*—All varieties. The bouillon becomes turbid, and there is a small white precipitate which in ten to fourteen days becomes dark grey.

4. *Saccharose Bouillon.*—All varieties. In five days the bouillon becomes turbid and there is a white precipitate which turns to grey.

5. *Saccharose Agar Stab.*—All the varieties grew except Bangalore. The growth reached the walls of the test-tube in four days in the case of Sind, 10 days Nagpur, 14 days Pusa and Cawnpore. The Darjeeling growth was much less.

6. *Mannite Soil Extract.*[1]—All varieties. A white precipitate was formed within six days; later this changes to grey and brown.

7. *Potato.*—Irregularity was observed in the growth of the several varieties. In the first inoculation the Bangalore variety produced a light yellowish brown growth in six days. This later turned to a dark brown. The inoculation was repeated. No growth occurred this time on the Bangalore variety, but the Cawnpore and Pusa varieties produced a yellow growth, which later turned chocolate colour. (Plate V.)

8. *Synthetic Potato.*—A white growth appeared in two days, and later became slightly brown. The growths of the several varieties differed slightly in consistency.

9. *Nitrate Broth.*—No visible growth, and no ammonia formation.

10. *Milk.*—Practically no change in three weeks, except that the colour of the milk became slightly browner.

11. *Litmus Milk.*—The blue colour became slightly deeper.

12. *Pigment Production.*—Practically all cultures turned brown with age. Black pigment occurred only in impure or contaminated cultures of the varieties examined.

STAINING.

Capsules.—Pl. I, fig. 5.

To demonstrate the presence of capsules, stain a smear from a colony three or four days old, grown on a mannite agar plate. Use carbol fuchsin or gentian violet.

---

[1] Aqueous extract of Soil + 2% mannite.

Flagella.—Pl. I, figs. 7 and 8.

To a watchglass containing about one c.c. of water and a drop of 2% osmic acid solution sufficient of a culture is added to make the liquid slightly turbid. A loopful of the liquid is spread over a coverslip and allowed to dry. The film on the coverslip is mordanted with Zettnow's mordant for 5—7 minutes; the mordant is allowed to cool, the coverslip is removed, washed in tap water, dried, covered with an ammoniacal solution of silver sulphate and warmed over a flame till the edges of the film blacken. The coverslip is washed in water, dried and mounted in Canada balsam. It is much more difficult to get a good preparation of *Azotobacter* than of most other motile organisms (such as *B. subtilis*).

Granules.—Pl. I, fig. 6 and Pl. II, figs. 2 and 7.

Azotobacter in young cultures about a day old stains evenly and deeply. From cultures four days old or more it shows deeply stained granules in a faintly stained or clear ground mass, with a fine meshlike structure. The best stains tried for demonstrating the presence of the granules and mesh are Jenner's, Leishmann's, and Giemsa's. Methylene blue is not quite so good. Carbol fuchsin stains deeply and the finer details are obscured.

In cultures 24 hours old, Jenner's stain often showed the presence of a single very deeply stained nucleus or granule. A fine thread may be seen joining a pair of cells that had just resulted from the division of a single cell. (Pl. II, fig. 8.)

Gram's Stain.

Cultures, twenty-four hours old or more, were examined, and the different varieties were always Gram positive.

Conclusion.

1. The nitrogen-fixing power of *Azotobacter* in cultures inoculated with Pusa soil compares very favourably with that observed by other investigators.[1]

2. There is a well marked seasonal variation in the nitrogen fixation in cultures inoculated with soil.

[1] Söhngen, *Cent. für Bakt.* Abt. *II*, Vol. XXXVIII, p. 630.
Sackett, *ibid* Vol. XXXIV, p. 100.
Rosing, *ibid* Vol. XXXIII, p. 619.
Remy and Rosing *Cent. für Bakt.* Abt. II, Vol. XXX, p. 349.
Hoffmann and Hammer *Cent. für Bakt.* Abt. II, Vol. XXVIII, p. 127.
H. H. Green, and Greave, *Cent. für. Bakt.* Abt. II, Vol. XLI, p. 577, and p. 444.

3. Pure cultures of *Azotobacter* isolated from different soils vary in nitrogen-fixing power and in morphological and cultural characters. These morphological and cultural characters are constant in any particular variety.

4. Black pigment occurred only in impure cultures.

5. Nitrogen fixation in soil is increased by cultivation and the addition of suitable carbohydrate material. We may conclude that proper soil management should include the provision of conditions favourable to the physiological activity of *Azotobacter*, namely aeration, the presence of lime,[1] and the presence of available carbohydrate food. The increased nitrogen fixation observed as resulting from the addition of humus, and the experimental demonstration by Koch[2] that cellulose may be acted on by micro-organisms in the soil so as to make it available as carbohydrate food for *Azotobacter*, emphasize the importance of such agricultural operations as tend to maintain the supply of organic matter in the soil.

PUSA,
February 10th, 1915.

[1] Christensen and Hansen, *Cent. für Bakt.* Abt. II, Vol. XXIX, p. 355; also Loew, "Studies on Acid Soils of Porto Rico," *Porto Rico Expt. Sta. Bull.* No. 13.

[2] *Cent. für Bakt.* Abt. II, Vol. XXVII, p. 1.

EXPLANATION OF PLATES.

Plate I.

Fig.	1.	× 1000	Impure culture, 3 days old, with Pusa soil as inoculum.
	2.	× 950	Azotobacter, Pusa, 24 hours old.
	3.	× 1000	,, ,, 7 days old.
	4.	× 1350	,, ,, 3 months old
	5.	× 735	,, ,, Capsules.
	6.	1980	,, Granules, Leishmann's stain.
	7.	× 900	,, Flagella.
	8.	× 735	Cawnpore Flagella.
	9.	× 1000	,, 24 hours old.
	10.	× 1000	,, 7 days old.

Plate II.

Fig.	1.	× 625	Azotobacter, Darjeeling, 24 hours old.
	2.	× 500	,, 7 days old.
	3.	× 1000	Bangalore, 24 hours old.
	4.	× 1000	,, 15 days old.
	5.	× 500	Nagpur, 24 hours old.
	6.	× 1000	,, 7 days old.
	7.	× 1000	,, 7 days old. Leishmann's stain.
	8.	× 1000	Sind, 24 hours old.
	9.	× 1000	,, 7 days old. Giemsa's stain.

Plate III. × $\frac{5}{9}$.

Fig.	1.	Azotobacter,	Pusa.	Colonies on mannite agar plate			3 days old.
,,	2.	,,	Bangalore	,,	,,	,,	,,
,,	3.	,,	Cawnpore	,,	,,	,,	,,
,,	4.	,,	,,	,,	,,	,,	9 days old.

Plate IV. × $\frac{5}{9}$.

Fig.	1.	Azotobacter,	Darjeeling.	Colonies on mannite agar plate			3 days old.
,,	2.	,,	,,	,,	,,	,,	9 days old.
,,	3.	,,	Sind	,,	,,	,,	4 days old.
,,	4.	,,	Nagpur	,,	,,	,,	4 days old.

Plate V. × $\frac{3}{4}$.

Azotobacter, Cawnpore, growth on potato, three weeks old.

Fig. 1.

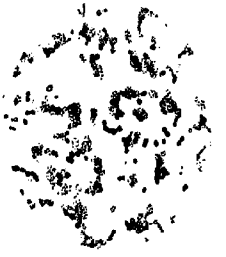


Fig. 2.

Fig. 3.

Fig. 4.

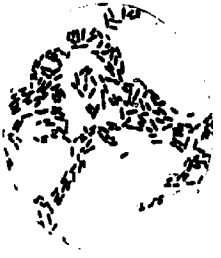


Fig. 5.

Fig. 6.

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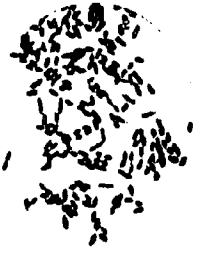


Fig. 8.

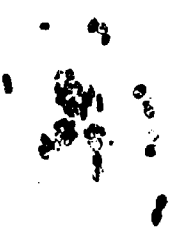


Fig. 9.

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.

Fig. 7.

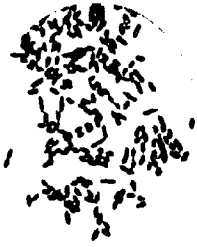
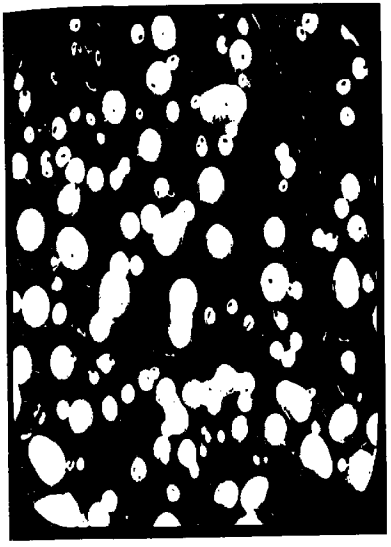


Fig. 8.

Fig. 9.



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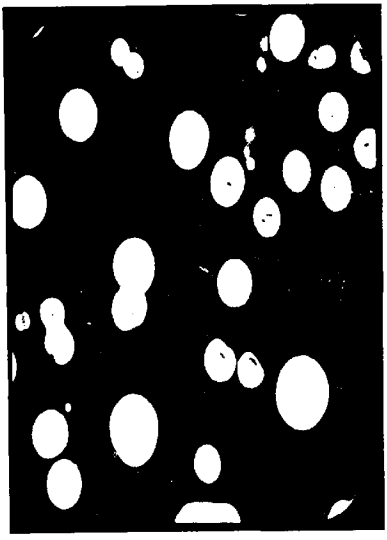


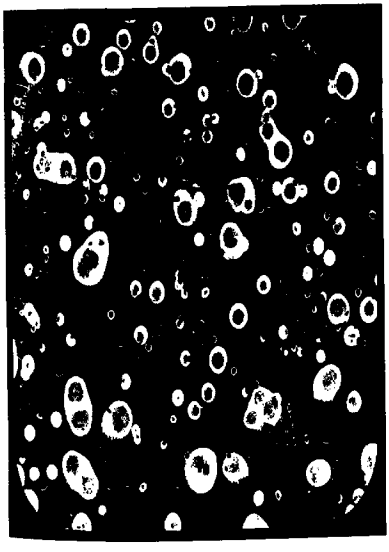


Fig. 3.

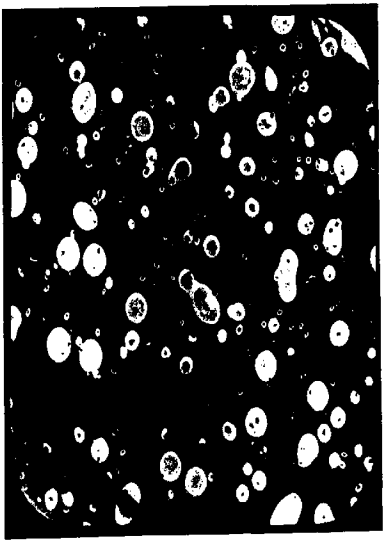


Fig. 4.

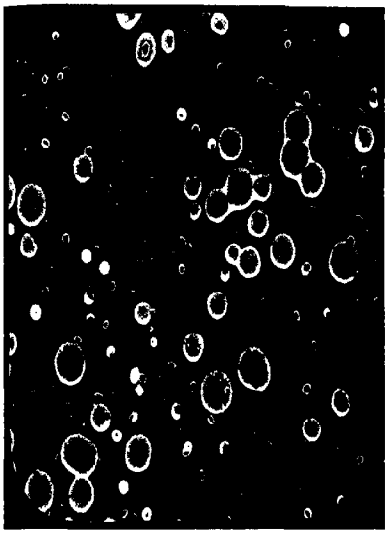
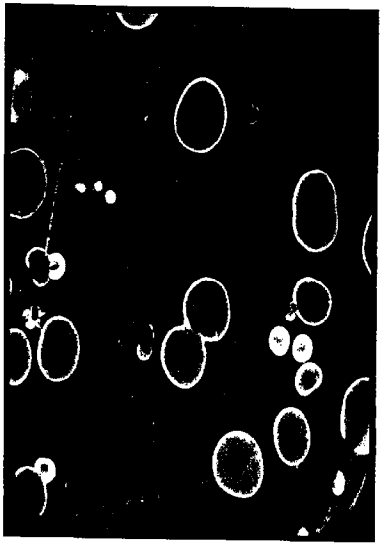


Fig. 1.



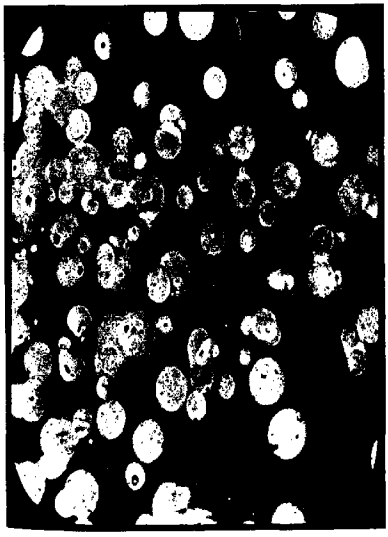


Fig. 3.

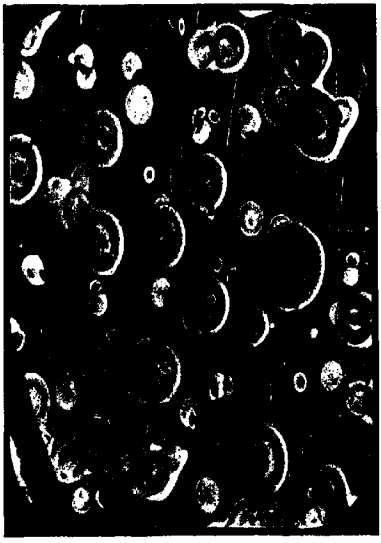


Fig. 4.

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